PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:	WO 99/15904
G01N 33/68, C07K 16/24, G01N 33/577	A1	(43) International Publication Date:	1 April 1999 (01.04.99)

(21) International Application Number: PCT/US98/20167

(22) International Filing Date: 25 September 1998 (25.09.98)

(30) Priority Data:
08/937,991 26 September 1997 (26.09.97) US

(71) Applicant: UNIVERSITY OF WASHINGTON [US/US]; Suite 200, 1107 N.E. 45th Street, Seattle, WA 98105 (US).

(72) Inventors: GIACHELLI, Cecilia, M.; 3012 154th Street, S.E., Mill Creek, WA 98012 (US). JOHNSON, Richard, J.; 100 Ward Street #204, Seattle, WA 98109 (US).

(74) Agents: GAY, David, A. et al.; Campbell & Flores LLP, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS AND COMPOSITIONS FOR DIAGNOSING RENAL PATHOLOGIES

(57) Abstract

The invention provides a method of diagnosing a renal pathology. The method consists of determining a concentration of urinary osteopontin in a sample isolated from an individual having or suspected of having a renal pathology and comparing the determined concentration to a urinary osteopontin concentration from a normal individual, wherein the determined concentration of urinary osteopontin in the sample indicates the presence or absence of the renal pathology. An increase in the determined concentration of urinary osteopontin in the sample compared to the urinary osteopontin concentration in a normal individual indicates the presence of the renal pathology. An unchanged concentration of urinary osteopontin in the sample compared to the urinary osteopontin concentration in a normal individual indicates the absence of a renal pathology. Methods of predicting the onset of a renal pathology and methods of monitoring the progression of a renal pathology are also provided. Additionally provided is a kit for diagnosing a renal pathology. The kit includes an osteopontin–selective antibody, osteopontin and an ancillary reagent.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHODS AND COMPOSITIONS FOR DIAGNOSING RENAL PATHOLOGIES

This invention was made with government support under grant number DK 47659 awarded by the National Institutes of Health. The United States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention relates generally to renal pathologies and, more specifically, to the diagnosis of renal pathologies through measurement of secreted urinary polypeptides.

The kidney is a vital organ which functions in mammals to filter blood and excrete waste products of metabolism. Decline or loss in kidney function leads to severe physiological disorders resulting in debilitation and even death.

There are a large number of different hereditary and physiological abnormalities which result in various degrees of kidney or renal pathologies. These pathologies range from congenital anomalies and cystic diseases of the kidney to acute and chronic renal failure. Acute renal failure refers to a sudden decline in glomerular filtration rate. In contrast, chronic renal failure typically results from a large variety of renal pathologies, including noninflammatory glomerulopathies and glomerulonephritis as well as tubulointerstitial and vascular diseases.

Acute and chronic renal pathologies which lead to a total loss of kidney function affect over 220,000 individuals in the United States at an estimated cost of

about 8.6 billion dollars per year. For example, regardless of etiology, most forms of chronic renal pathologies progress to end stage glomerulosclerosis and tubulointerstitial fibrosis with a corresponding loss of kidney function. Moreover, end stage renal failure manifests itself further as complicated disorders of multiple organ systems, including the skeletal, cardiopulmonary, hematopoietic and gastrointestinal systems. Therefore, unless early diagnosis and treatment occurs, many end stage renal pathologies will require additional medical treatments and patient care to address the associated complications of multiple organ system disorders. Nevertheless, the end result is an additional reduction in the length and quality of life.

Current treatments for renal failures include life-long kidney dialysis and kidney transplantation. Dialysis is time consuming, expensive and generally results in only a moderate improvement of the quality of life. In contrast, kidney transplantation is a potential treatment which can result in a significant improvement in the quality of life. However, this treatment is only available to the limited few who find donors that have a compatible tissue antigen match so as to avoid the potential risk of graft rejection.

The primary test for renal function measures serum creatinine levels. One drawback, however, is that this test is relatively insensitive and will only assess current renal function. Serum creatinine levels do not provide an indication of whether there is any ongoing renal damage.

Assessment of chronic renal pathologies has traditionally been performed by renal biopsy.

Unfortunately, renal biopsies are invasive and therefore associated with significant medical risks. Moreover, as with most surgical procedures, renal biopsies are also expensive procedures to perform and are additionally associated with further procedures including processing and analysis of the sample. Each of these associated procedures also require trained medical and professional personnel and incur costs that are ultimately passed on to the patient.

Other procedures are also known which have been used for assessing renal function. For example, urinary microalbuminuria has been used as a potential indicator of early diabetic renal injury, and to a lesser extent, an indicator of early hypertensive injury. Similarly, urinary β2 microglobulin has been used as a potential measure of tubular function. Unfortunately, these assays are limited to the measurement of glomerular damage and tubular function and are therefore unapplicable to many chronic renal pathologies such as those related to tubulointerstitial injuries. Other proteins have also been examined for potential utility in marking progressive renal disease. However, the sensitivity and accuracy required for reliably predicting renal pathologies using these other markers has not been achieved.

Thus, there exists a need for simple, accurate and sensitive methods which can reliably predict the occurrence or progression of renal pathologies. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a method of diagnosing a renal pathology. The method consists of determining a concentration of urinary osteopontin in a sample isolated from an individual having or suspected of having a renal pathology and comparing the determined concentration to a urinary osteopontin concentration from a normal individual, wherein the determined concentration of urinary osteopontin in the sample indicates the presence or absence of the renal pathology. An increase in the determined concentration of urinary osteopontin in the sample compared to the urinary osteopontin concentration in a normal individual indicates the presence of the renal pathology. An unchanged concentration of urinary osteopontin in the sample compared to the urinary osteopontin concentration in a normal individual indicates the absence of a renal pathology. Methods of predicting the onset of a renal pathology and methods of monitoring the progression of a renal pathology are also provided. Additionally provided is a kit for diagnosing a renal pathology. The kit includes an osteopontin-selective antibody, osteopontin and an ancillary reagent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) for human osteopontin according to Young et al., Genomics 7:491-502 (1990).

Figure 2 shows an immunoblot analysis for osteopontin levels in urine isolated from normal animals and from animals exhibiting interstitial inflammation and fibrosis of the kidney.

Figure 3 shows the quantitation of human osteopontin samples using an ELISA assay.

Figure 4 shows a standard curve of osteopontin and the level of osteopontin found in normal urine and urine from patients with renal damage.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to simple and accurate methods which reliably diagnose the occurrence of renal pathologies. The methods are applicable to the diagnosis of a variety of pathologies including those involving inflammatory glomerular lesions, tubulointerstitial diseases and vascular diseases. The methods are similarly applicable to age-related renal disorders and noninflammatory glomerulopathies. One advantage of the methods of the invention is that they measure urine concentrations of a marker polypeptide and therefore allow a rapid and non-invasive procedure for diagnosing a renal pathology. Another advantage of the methods of the invention is that they also are applicable for predicting the onset or measuring the progression of a renal pathology.

In one embodiment, the invention is directed to the measurement of urinary levels of osteopontin as an indicator for assessing the presence or extent of a renal pathology. The methods are based on the discovery that the changes in urine osteopontin levels correlate significantly with the occurrence and severity of renal pathologies. High levels of urinary osteopontin indicate the occurrence of a renal pathology. Osteopontin levels are measured using an immunoaffinity based assay which is amenable to multisample formats and automation.

As used herein, the term "renal pathology" is intended to mean an aberrant structural or functional change in cells or tissues within the kidney which lead to a loss or decline in renal function. Such aberrant structural or functional changes result in an increased and abnormal secretion of osteopontin into the urine. Renal pathologies include, for example, aberrant renal conditions falling within the categories of congenital anomalies, cystic diseases, glomerular syndromes, noninflammatory glomerulopathies, glomerulonephritis, tubulointerstitial diseases and vascular diseases.

As used herein, the term "osteopontin" is intended to mean the cell adhesion protein originally identified as bone sialoprotein I and has since been determined to be an ubiquitous component of connective tissue. Osteopontin has now been found to be produced by numerous cell types other than the osteoclasts and osteoblasts of bone and is also known in the art as uropontin, secreted phosphoprotein I, 2B7 and Eta 1 (Butler, W.T., Connect. Tissue Res. 23:123-136 (1989)). The polypeptides encompassed by all of these terms used in the art are included within the definition of osteopontin as used herein.

Osteopontin is an extracellular matrix protein containing heparin and calcium binding sites. Osteopontin also contains the Arg-Gly-Asp (RGD) cell adhesion sequence and a thrombin cleavage site. Osteopontin exhibits binding activity to RGD binding integrins such as $\alpha v\beta 3$, $\alpha v\beta 1$ and $\alpha v\beta 5$ as well as to the cell surface protein CD44. The nucleotide and deduced amino acid sequence for osteopontin has been described by Young et al., *supra*, 1990, and is set forth herein as Figure 1 (SEQ ID NOS:1 and 2). Further, multiple

isoforms of osteopontin exist due to differential splicing of the mRNA (Young et al., supra, 1990). Osteopontin has a calculated molecular weight of 34 kilodaltons and an apparent molecular weight of 44-66 kilodaltons as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The term osteopontin is intended to include polypeptides having substantially the same amino acid sequence as shown as SEQ ID NO:2 and encoded by the nucleotide sequence shown as SEQ ID NO:1. Modifications of the polypeptide sequences shown in SEQ ID NO:2 and encoded by SEQ ID NO:1 are included within the definition so long as the modified forms maintain their osteopontin identifiable characteristics. Modified forms include, for example, post-translational modifications such as phosphorylation, glycosylation, acylation and truncation. Additionally, the term is intended to include derivatives and variants such as the various osteopontin isoforms as well as variants produced by mutation in an osteopontin-expressing host so long as such derivatives and variants maintain indicative features which selectively identify them as osteopontin polypeptides.

Fragments that substantially correspond to osteopontin and as such can be used as authentic measurements of osteopontin in a urine sample are similarly included within the definition of the term as used herein. Such fragments include, for example, immunogenic portions of osteopontin that maintain their binding reactivity and specificity for osteopontin-selective antibodies. Specific examples of such osteopontin fragments include, for example, osteopontin isoforms, osteopontin containing posttranslational modifications such as phosphorylation and glycosylation

as well as thrombin cleavage fragments and breakdown products which occur in the urine. All of such forms as well as others known to those skilled in the art are intended to be encompassed by the definition of the term as used herein so long as such forms can be selectively identified as an osteopontin polypeptide.

As used herein, the term "affinity binding assay" is intended to mean a binding assay which selectively distinguishes an analyte by affinity or avidity. Affinity binding assays are well known in the art and include, for example, antigen-antibody interactions and receptor-ligand interactions. Such assays can be performed using a solid phase, for example, to immobilize one component of the binding reaction as well as in solution or *in situ*. An "immunoaffinity binding assay" as used herein, is a specific type of affinity binding assay which uses binding molecules of the immunoglobulin superfamily of polypeptides. Such polypeptides can include, for example, antibodies, T cell receptors, CD4, and CD8 as well as others known to those skilled in the art. Specific examples of immunoaffinity binding assays include, for example, all formats known in the art for enzyme-linked immunosorbant assays (ELISA), radioimmune assays (RIA) and immunoblot analysis.

As used herein, the term "antibody" when used in reference to an immunoaffinity assay is intended to mean a population of immunoglobulin molecules having a characteristic binding specificity toward a particular antigen. The population of immunoglobulin molecules can be polyclonal or monoclonal and of any isotype. The term is also intended to include functional fragments and modified forms thereof which maintain substantially the same binding specificity to the particular antigen. Such

functional fragments include, for example, Fab, $(Fab)_2$, Fv, single chain Fv (scFv) and the like and are well known to those skilled in the art. Complementarity determining regions (CDR) sequences that maintain substantially the same binding specificity as the functional antibody are similarly included within the definition of the term as used herein. The term is similarly intended to include recombinantly produced antibodies and modified forms such as chimeric, humanized, bifunctional and the like. Modifications such as chemical moieties, detectable labels, linkers and other attached functional agents are similarly included within the meaning of the term so long as the antibody maintains its binding specificity to the particular antigen.

As used herein, the term "selective" when used in reference to an antibody or other osteopontin binding molecule is intended to mean an antibody or other osteopontin binding molecule, or functional fragment thereof which does not substantially cross react, or can be made to not substantially cross react with non-osteopontin related polypeptides.

As used herein, the term "ancillary reagent" when used in reference to a kit is intended to mean any reagent that aids in the preparation or is useful for performing the osteopontin detection methods of the kit. Ancillary reagents can therefore be biological and chemical reagents such as isolated polypeptides which are used as controls or standards, chemical reactants, buffers and the like. Ancillary reagents can similarly be inert materials such as written instructions for performing the methods of the kit. Thus, the term is intended to include all reagents, instructions, materials

and props that can be useful in performing the osteopontin detection methods of the invention.

The principal causes of renal failure include diabetes, hypertension, glomerulonephritis and polycystic kidney disease (U.S. Renal Data System, 1996 Annual Data Report, The National Institute of Health, USRDS Coordination Center, University of Michigan, Ann Arbor, MI). Hypertension is also associated with tubulointerstitial and vascular injury of the kidney (Sommers et al., Am. J. Pathol. 34:685-714 (1958)). Age-related renal pathologies similarly involve increased tubulointerstitial fibrosis and tubular injury. Irrespective of etiology, all of these renal diseases are characterized by progressive scarring of the renal interstitium (interstitial fibrosis). It is the degree of interstitial fibrosis that best predicts the degree of renal dysfunction as well as the risk for progression to end stage.

In the normal kidney, osteopontin is minimally expressed in the renal cortex, being expressed by only occasional distal tubular cells and parietal epithelium. Similarly, in the renal medulla, low levels osteopontin are expressed in the loops of Henle and deeper in the medullary collecting duct (Giachelli et al., Kidney Int. 45:515-524 (1994); Kleinman et al., Kidney Int. 47:1585-1596 (1995)).

In an animal model of tubulointerstitial injury and inflammation, however, osteopontin expression and accumulation is observed to be increased and can be correlated with the areas of injury as well as with the localization of macrophages at the sites of injury. Based, in part on these observations and the known cell

adhesive properties of osteopontin, it has been suggested that the increased osteopontin accumulation observed in renal interstitial injuries functions to recruit macrophages to the site of the injury. The macrophage infiltration is further thought to be responsible for further local injury and fibrosis of the kidney (Pichler et al., Miner Electrolyte Metab. 21:317-327 (1995).

Osteopontin has similarly been shown to exhibit increased expression and accumulation in other models of renal pathologies. Such models include, for example, animal models of glomerulonephritis and nephritis (Pichler et al., Am. J. Pathol. 144:915-926 (1994); Eddy and Giachelli, Kidney Int. 47:1546-1557 (1995); Lan et al., J. Am. Soc. Nephrol. 7:1738 (1996)). Osteopontin expression is also increased at sites of tubulointerstitial inflammation in other models, including streptozotocin-induced diabetes, cyclosporine nephropathy, ureteral obstruction, renal ischemia, in polycystic kidneys and with the tubulointerstitial inflammation observed in aging rats. Similarly, osteopontin is expressed by tubules during the recovery from acute renal failure caused by various toxins.

Similar to the animal models described above, in normal human kidney there is very little basal osteopontin expression in the cortex. However, in glomerulonephritis, osteopontin is increased in tubules and correlates with macrophage infiltration ($p<0.0001$), fibrosis ($P<0.0002$), and serum creatinine (p 0.008), as observed in the animal model of renal pathologies (Barisoni et al., J. Am. Soc. Nephrol. 7:1751 (1996)). Human individuals with essential hypertension have marked increases in osteopontin expression in their tubules compared to normal subjects.

In all of the above models and human individuals, any correlation of osteopontin expression has been limited to its association and role in promoting macrophage infiltration and tubular injury. There has not been any correlation of osteopontin expression in diseased renal tissue with its concentration as a waste product in urine.

The invention provides a method of diagnosing a renal pathology. The method consists of determining a concentration of urinary osteopontin in a sample isolated from an individual having or suspected of having a renal pathology and comparing the determined concentration to a urinary osteopontin concentration from a normal individual, wherein the determined concentration of urinary osteopontin in the sample indicates the presence or absence of the renal pathology. An increase in the concentration of urinary osteopontin in the sample isolated from the individual having or suspected of having a renal pathology compared to the urinary osteopontin concentration from a normal individual indicates the presence of the renal pathology. An unchanged concentration of urinary osteopontin in the sample isolated from the individual having or suspected of having a renal pathology compared to the urinary osteopontin concentration from a normal individual indicates the absence of a renal pathology.

The methods of the invention are directed to the finding of a positive and significant correlation between osteopontin concentrations in urine and the degree or extent of a renal pathology. The diagnostic methods of the invention are applicable to essentially any renal pathology. Such renal pathologies include, for example, congenital anomalies, cystic diseases,

glomerular syndromes, noninflammatory glomerulopathies, glomerulonephritis, tubulointerstitial diseases and vascular diseases. A consequence of these aberrant renal conditions is that there is a concomitant aberrant secretion of increased levels of osteopontin into the urine. These aberrantly increased urinary concentrations of osteopontin can therefore be used as a diagnostic indicator of renal pathologies and loss of renal function in essentially all mammals, including human.

Assessing urinary osteopontin concentrations is also a predictive indication of the degree or severity of a renal pathology since increased concentrations positively correlate with increased severity of the pathology. Conversely, a regression of the severity of a renal pathology is followed by a corresponding decrease in the urinary concentrations of osteopontin.

Therefore, the invention provides a method of diagnosing a renal pathology wherein the pathology is selected from a group of renal pathologies consisting of tubulointerstitial inflammation, tubulointerstitial injury, glomerulonephritis, polycystic kidney disease, hypertensive renal damage, diabetes, renal ischemia, ureteral obstruction, cyclosporine toxicity, age-related renal pathologies, renal fibrosis and renal failure.

The use of urinary osteopontin concentrations as a diagnostic indicator of a renal pathology allows for early diagnosis as a predictive indicator when no physiological or pathological symptoms are apparent. The methods are applicable to individuals with familial history of a renal pathology or predicted to be at risk by reliable prognostic indicators prior to onset of overt clinical symptoms. All that is necessary is to determine

the urinary concentrations of osteopontin to determine whether there is a significant increase in osteopontin levels in the individual suspected of having a renal pathology compared to normal individuals.

Therefore, the invention provides a method of predicting the onset of a renal pathology. The method consists of determining an increased concentration of urinary osteopontin in a sample isolated from an individual having or suspected or having a renal pathology compared to a sample isolated from a normal individual, wherein the increased concentration of urinary osteopontin in the sample indicates the onset of the renal pathology.

Normal human individuals contain concentrations of urinary osteopontin between about 0-3 µg/ml (Bautista et al. J. Cellular Biochem. 61:402-409 (1996)). For example, the level of urinary osteopontin has been shown to be about 3800 ± 1800 µg/24 hr or about 1.9 µg/ml (Min et al., Kidney International 53:189-193 (1998)). On average, however, a concentration of about 2.5 µg/ml of osteopontin in the urine can be reliably used as the osteopontin concentration for a normal individual. The exact levels of osteopontin will vary depending on, for example, the hydration state of the individual and dilution of the urine. Such variations are known to those skilled in the art and can be accounted for when assessing the meaning of a measured osteopontin concentration. Concentrations of urinary osteopontin that are predictive of a renal pathology are therefore greater than about 5 µg/ml. However, increased concentrations greater than about 10-30 µg/ml are preferable with concentrations greater than about 40

µg/ml being more preferable. Nevertheless, urinary osteopontin concentrations of greater than about 20 µg/ml are in general applicable as a diagnostic indicator of a renal pathology. Similar relative increases in urine osteopontin concentrations are applicable to individuals of non-human species.

Urinary osteopontin concentrations can similarly be used to monitor the progression of a renal pathology. Two samples acquired at different time points are required to be analyzed for an assessment of progression or regression of a renal pathology. A significant increase in urinary osteopontin concentrations over time indicates progression of the renal pathology. In contrast, a significant decrease in urinary osteopontin concentrations over time indicates regression of the renal pathology.

Significant increases in osteopontin concentration between a first urine sample and a sample isolated at a later time point includes, for example, a change in urinary osteopontin concentration of greater than at least about 5 µg/ml. However, increases greater than about 10 µg/ml are preferable with changes being greater than about 30 µg/ml being more preferable. Nevertheless, increases in urinary osteopontin concentrations of about 20 µg/ml are in general applicable as a diagnostic indicator of the progression of a renal pathology. Conversely, corresponding decreases in the above urinary osteopontin concentrations between a first and temporally later sample from the same individual is indicative of a regression of the renal pathology.

Therefore, the invention provides a method of monitoring the progression of a renal pathology. The method consists of (a) determining the concentration of urinary osteopontin in a first sample isolated from an individual having or suspected of having a renal pathology; (b) determining the concentration of urinary osteopontin in a temporally independent second sample isolated from the individual; and (c) comparing the concentration of urinary osteopontin in the first and second samples from the individual, wherein an increase in the osteopontin concentration between the first and the second samples is indicative of an increased progression of the renal pathology and wherein a decrease in the osteopontin concentration between the first and second samples is indicative of a decreased progression of the renal pathology.

For all of the above-described diagnostic applications, accuracy, sensitivity and reliability can be further increased by standardizing the measured urinary osteopontin concentrations. Standardization will normalize the measured osteopontin values to account for the hydration status and metabolic state of the individual or the dilution of the urine. For example, the measured urinary osteopontin values can be standardized to urinary creatinine concentrations because steady state total urinary creatinine excretion is relatively constant. Such standardization is well known to those skilled in the art.

Any of a variety of methods known to those skilled in the art can be used for measuring or determining urinary osteopontin concentrations in an individual having or suspected of having a renal pathology. For example, affinity binding assays using

probes that selectively bind to osteopontin are applicable for measuring the concentrations or amounts of osteopontin in a urine sample isolated from an individual. Other means of measuring the amount or concentration of a polypeptide in a sample are similarly applicable for the diagnostic methods of the invention. Such other means include, for example, chromatographic, electrophoretic and by mass analysis.

A variety of well known methods for each of the above categories are applicable for quantitative and/or qualitative determination of an analyte in a sample of interest. Such other methods are amenable for the analysis of many samples and include methods to assay individual samples or multiple samples simultaneously, for example, in a multi-well format or in multiple wells of a gel.

Specific examples of chromatographic methods applicable for use in determining the concentration of urinary osteopontin include, for example, thin layer and gas chromatography. Electrophoretic methods include one and two dimensional electrophoresis as well as capillary electrophoresis. Osteopontin concentrations can be determined, for example, by staining with a dye or by blot analysis with a selective binding reagent. Mass spectrometry is one approach to determining the presence of osteopontin in a urine sample by mass analysis. Numerous other methods known to those skilled in the art exist which are applicable for determining the concentration of osteopontin in a urine sample. Such other methods can be employed equally in the methods of the invention for the diagnosis, prediction or monitoring of a renal pathology.

Essentially all modes of affinity binding assays are applicable for use in the methods of the invention. Such methods are rapid, efficient and sensitive. Moreover, affinity binding methods are simple and can be adjusted to be performed under a variety of clinical settings and conditions to suit a variety of particular needs. Affinity binding assays which are known and can be used in the methods of the invention include both soluble and solid phase formats. A specific example of a soluble phase affinity binding assay is immunoprecipitation using an osteopontin-selective antibody or other binding molecule. Solid phase formats can be advantageously used in methods of the invention since they are rapid and can be performed more easily on multiple different samples simultaneously without loosing sensitivity or accuracy. Moreover, solid phase affinity binding assays are further amenable to high throughput screening and automation. Thus, the invention provides a method of diagnosing a renal pathology, wherein a urinary osteopontin concentration is determined by an affinity binding assay.

Specific examples of solid phase affinity binding assays include immunoaffinity binding assays such as enzyme-linked immunosorbant assay (ELISA) and radioimmune assay (RIA). Other solid phase affinity binding assays are known to those skilled in the art and are applicable to the methods of the invention. Although affinity binding assays are generally formatted for use with an antibody binding molecule that is selective for the analyte or ligand of interest, essentially any binding molecule can be alternatively substituted for the selectively binding antibody in such affinity binding assays. Such binding molecules include, for example, macromolecules such as polypeptides, peptides, nucleic

acids, lipids and sugars as well as small molecule compounds. Methods are known in the art for identifying such molecules which bind selectively to a particular analyte or ligand and include, for example, surface display libraries and combinatorial libraries. Thus, for a molecule other than an antibody to be used in an affinity binding assay, all that is necessary is for the binding molecule to exhibit selective binding activity for the analyte or ligand of interest. Therefore, in the methods of the invention, all that is necessary is for the binding molecule to exhibit selective binding activity for osteopontin.

Various modes of affinity binding assays are similarly known which can be used in the diagnostic methods of the invention. For the purpose of illustration, particular embodiments of such affinity binding assays will be described further in reference to immunoaffinity binding assays. The various modes of affinity binding assays such as immunoaffinity binding assays include, for example, solid phase ELISA and RIA as well as modifications thereof. Such modifications thereof include, for example, capture assays and sandwich assays as well as the use of either mode in combination with a competition assay format. The choice of which mode or format of immunoaffinity binding assay to use will depend on the intent of the user. Such methods can be found described in common laboratory manuals such as Harlow and Lane, Antibodies: A Laboratory Manual New York: Cold Spring Harbor Laboratory (1988).

A specific example of an immunoaffinity binding assay for quantitating the amounts or concentrations of osteopontin in a urine sample is described further below

in Example II. This Example describes the use of a capture ELISA assay which employs a first osteopontin-selective antibody which selectively captures osteopontin from the urine sample and immobilizes it onto a solid support. Following selective capture of osteopontin from the urine sample, a second osteopontin-selective antibody is employed to measure the amount of bound osteopontin and therefore the concentration of osteopontin in the urine sample. Detection of the bound second osteopontin-selective antibody is performed using, for example, a labeled secondary antibody to the osteopontin-selective antibody.

The affinity binding assays of the invention, can be used in conjunction with a variety of detection labels and systems known in the art to quantitate the osteopontin concentrations in the analyzed urine samples. Detection systems include the detection of bound osteopontin by both direct and indirect means. For example, direct detection of a bound osteopontin-selective antibody, or binding molecule, can be performed by directly labeling the osteopontin-selective antibody that is used for quantitating the amount of osteopontin in a sample. Indirect detection systems include, for example, the use of labeled secondary antibodies and other binding molecules or the use of such antibodies and binding molecules that are conjugated to a further moiety which can be selectively bound by a detectable label. A specific example of such a moiety that can be selectively bound by a detectable label is biotin which can be bound by an avidin or a streptavidin conjugated label.

Secondary antibodies, labels and detection systems are well known in the art and can be obtained commercially or by techniques well known in the art. The

detectable labels and systems employed in the osteopontin-selective binding assays of the invention should not impair binding of the osteopontin-selective antibody to osteopontin. Moreover, multiple antibody and label systems can be employed for detecting the bound osteopontin-selective antibody to enhance the sensitivity of the binding assay if desired. Such detection molecules and systems are well known in the art and can also be found described in, for example, Harlow and Lane, *supra*.

Detectable labels can be essentially any label that can be quantitated or measured by analytical methods. Such labels include, for example, enzymes, radioisotopes, fluorochromes as well as chemi- and bioluminescent compounds. Specific examples of enzyme labels include horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase, urease and luciferase.

A horseradish-peroxidase detection system can be used, for example, with the chromogenic substrate tetramethylbenzidine (TMB), which yields a soluble product in the presence of hydrogen peroxide that is detectable by measuring absorbance at 450 nm. An alkaline phosphatase detection system can be used with the chromogenic substrate *p*-nitrophenyl phosphate, for example, which yields a soluble product readily detectable by measuring absorbance at 405 nm. Similarly, a β-galactosidase detection system can be used with the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG), which yields a soluble product detectable by measuring absorbance at 410 nm, or a urease detection system can be used with a substrate such as urea-bromocresol purple (Sigma Immunochemicals, St. Louis, MO). Luciferin is the substrate compound for

luciferase which emits light following ATP-dependent oxidation.

Fluorochrome detection labels are rendered detectable through the emission of light of ultraviolet or visible wavelength after excitation by light or another energy source. DAPI, fluorescein, Hoechst 33258, R-phycocyanin, B-phycoerythrin, R-phycoerythrin, rhodamine, Texas red and lissamine are specific examples of fluorochrome detection labels that can be utilized in the affinity binding assays of the invention. A particularly useful fluorochrome is fluorescein or rhodamine.

Chemiluminescent as well as bioluminescent detection labels are convenient for sensitive, non-radioactive detection of osteopontin and can be obtained commercially from various sources such as Amersham Lifesciences, Inc. (Arlington Heights, IL).

Radioisotopes can alternatively be used as detectable labels for use in the osteopontin binding assays of the invention. Iodine-125 is a specific example of a radioisotope useful as a detectable label.

Signals from detectable labels can be analyzed, for example, using a spectrophotometer to detect color from a chromogenic substrate; a fluorometer to detect fluorescence in the presence of light of a certain wavelength; or a radiation counter to detect radiation, such as a gamma counter for detection of iodine-125. For detection of an enzyme-linked secondary antibody, for example, a quantitative analysis of the amount of bound osteopontin-selective antibody can be made using a spectrophotometer such as an EMAX Microplate Reader

(Molecular Devices, Menlo Park, CA) in accordance with the manufacturer's instructions. If desired, the assays of the invention can be automated or performed robotically, and the signal from multiple samples can be detected simultaneously.

The assays of the present invention can be forward, reverse or simultaneous as described in U.S. Patent No. 4,376,110 and No. 4,778,751. For example, an antigen of interest such as osteopontin can be detected in a sandwich-type assay, where two antibodies are used to bind and detect the presence of osteopontin. A first osteopontin-selective antibody can be attached to a solid support to capture osteopontin, and a second osteopontin-selective antibody can be used to detect the presence of osteopontin. Alternatively, a sample containing osteopontin can be initially incubated with the osteopontin-selective antibody used for detection and subsequently incubated with a capture antibody. In addition, a sample containing osteopontin can be incubated simultaneously with both capture and detection osteopontin-selective antibodies. It should be understood that, in a sandwich-type assay, any order or combination of osteopontin-selective antibodies can be used so long as both antibodies can bind to osteopontin and result in both capture and detection of osteopontin.

Separation steps for the various assay formats described herein, including the removal of unbound secondary antibody, can be performed by methods known in the art (Harlow and Lane, *supra*). For example, washing with a suitable buffer can be followed by filtration, aspiration, vacuum or magnetic separation as well as by centrifugation.

Thus, the invention provides a method of diagnosing a renal pathology. The method consists of the steps of: (a) contacting an immobilized first osteopontin-selective antibody with a urine sample isolated from an individual having or suspected of having a renal pathology under conditions which allow binding of osteopontin to the immobilized osteopontin-selective antibody so as to immobilize osteopontin in the urine sample; (b) contacting the immobilized osteopontin with a second osteopontin-selective antibody under conditions which allow binding of the second osteopontin-selective antibody to the immobilized osteopontin; and (c) determining the amount of bound osteopontin to the second osteopontin-selective antibody, wherein an increased amount of bound osteopontin from the urine of the individual having or suspected of having a renal pathology compared to bound osteopontin from urine of a normal individual indicates the presence of a renal pathology.

Methods of the invention were used to determine the level of osteopontin in human urine. Specifically, an ELISA was used to measure the level of osteopontin in normal urine and urine from patients with renal damage (see Example III).

Osteopontin in a urine sample was initially captured with an antibody specific for osteopontin. Various capture antibodies, including LF-7, LF-123 and LF-124, provided by Dr. Larry Fisher of the National Institutes of Health, were coated onto microtiter plates and incubated overnight. Any unbound antibody was washed away, and any unbound sites on the surface of the plate were blocked with bovine serum albumin. Antigen solutions, either known concentrations of recombinant

osteopontin or urine samples, were incubated on the plates. Osteopontin in the urine was bound by the capture antibodies, and excess protein and urine solutions were washed away. A primary antibody that binds to osteopontin such as the goat polyclonal antibody OP-189 was then added to the plates to bind osteopontin captured on the plate. A secondary antibody was used to detect the presence of osteopontin antibody, for example, biotinylated goat polyclonal antibody to detect OP-189. A chemical detection system was used to detect and quantify the amount of bound secondary antibody.

By quantifying the amount of secondary antibody bound to the plate and comparing to known concentrations of osteopontin, the concentration of osteopontin in human urine samples was determined. Normal human urine was found to have a concentration of about 4 to 6 µg/ml, whereas urine from patients with renal damage was found to be about five times greater, about 20 to 30 µg/ml (see Example III). These results indicate that methods of the invention directed to determining the concentration of urinary osteopontin are useful for diagnosing a renal pathology.

The invention additionally provides a kit for diagnosing a renal pathology. The kit consists of an osteopontin-selective antibody, osteopontin and an ancillary reagent.

Components for any of the above-described affinity binding assays can be assembled into a package such as a kit for the diagnosis or monitoring of a renal pathology. Immunoaffinity assays are particularly useful and practical as kits since they can be conveniently employed in essentially any desired clinical setting. An

immunoaffinity binding kit comprises reagents for the detection of osteopontin levels in a sample isolated from an individual having or suspected of having a renal pathology. Such reagents include, for example, an osteopontin-selective binding reagent such as an osteopontin-selective antibody and osteopontin which can be used as a positive control. The osteopontin can also be used for the construction of a standard which is used for quantitation osteopontin present in the urine sample.

Additional components of osteopontin detection reagents that can be included in the diagnostic kits of the invention include ancillary reagents such as binding buffers, dilution buffers and wash buffers. Detectable labels, detectable secondary antibodies or binding molecules and the like can similarly be packaged in the kits for quantitating the amount or concentration of urinary osteopontin present in a sample. Additionally, reactants and solutions for quantitating detectable labels can also be packaged into the osteopontin detection kits of the invention. Such reactants include, for example, any of the various enzyme substrates described previously. Solutions required for detecting labels include, for example, scintillation fluid for radioactive labels. Finally, the kits of the invention can additionally include written instructions for performing the osteopontin-binding assays of the invention in single or multiple sample format.

Concentrations and amounts of the various reactants and ancillary reagents will depend on the intended use and format of the kit. For example, a kit for use in a multiwell ELISA format will contain enough reagents to assay a specified number of samples. Typically, the number will coincide with a multiple of

the number of wells in the ELISA plate to be used but can include more or less reactants depending on the needs of a particular assay. Such concentrations and amounts are known to those skilled in the art and can be determined by the individual or entity packaging the reagents into the kits of the invention.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Correlation of Urinary Osteopontin Levels with Renal Pathologies

This Example demonstrates the correlation of urinary osteopontin levels with the degree of interstitial inflammation and fibrosis in an animal model of progressive renal pathologies.

The rat model of angiotensin II-induced hypertension was used to correlate levels of osteopontin in the urine with the degree of interstitial inflammation and fibrosis. This model is considered to be a credible animal model that is predictive for human patients with progressive renal pathologies. Osteopontin levels have been shown by histological evaluation in this model to be elevated in renal tissue derived from angiotensin II-treated rats compared to control treated rats. The increased levels of osteopontin similarly correlated with an increase in macrophage accumulation (Giachelli et al. Kidney Int. 45:515-524 (1994)).

This study shows that the elevated synthesis of osteopontin in the kidney leads to a corresponding increase in the levels of osteopontin secreted into the urine. Briefly, the study was performed as follows. Interstitial inflammation and fibrosis was induced in rats by treatment with angiotensin II. Male Zivic Miller Sprague Dawley rats (3 months old; 475-525 g) were treated continuously for 2 weeks with angiotensin II (200 ng/minute) by subcutaneous osmotic minipump infusion and maintained on the indicated diet for six weeks. Group A received a low salt diet (.002-.006% NaCl) for the two weeks of angiotensin II treatment, followed by a low salt diet for an additional 4 weeks. Group B received a low salt diet for the two weeks of angiotension II treatment, followed by a high salt diet (4% NaCl) for an additional 4 weeks. Group C received a high salt diet for the two weeks of angiotension II treatment, followed by a low salt diet for an additional 4 weeks. Group D received a high salt diet for the two weeks of angiotension II treatment, followed by a high salt diet for an additional 4 weeks. Group E served as normal controls and were not treated with angiotension II. Groups A to D developed interstitial disease as assessed by histology at 17 days and 6 weeks. Normal controls (Group E) showed no evidence of disease.

Urine samples were collected and processed as follows. A urine sample was collected overnight from each rat the day before sacrifice at six weeks. Rats were placed in a metabolic cage overnight, and urine was collected in a cup incubated on blue ice and containing 50 µl protease inhibitor solution (0.3 mM aprotinin, 1 mM leupeptin and 100 mM phenylmethylsulfonyl fluoride in 90% isopropanol). After collection, samples were frozen at -20°C until needed. Before processing, samples were

thawed at 4°C and centrifuged at 10,000 rpm for 15 minutes at 4°C. The urine samples were then transferred to Vp-10 concentrators with a molecular weight cut-off of 7500 (Vivapore; Vivascience; Acton MA) and allowed to concentrate overnight at 4°C. After recovering the samples, the final volumes were equalized by addition of PBS.

Osteopontin levels in the rat urine were quantitated by immunoblot analysis of the concentrated samples. Samples were prepared for western blotting by adding an equal volume of 2X Laemmli sample buffer to an amount of sample equivalent to 1 ml of the initial urine volume. Samples were reduced with dithiothreitol at a final concentration of 10 mM and boiled for 5 minutes, electrophoresed on 12.5% acrylamide and transferred to polyvinylidine difluoride (PVDF) membranes. Osteopontin levels were analyzed by immunoblotting with biotinylated OP199, an antibody that recognizes rat osteopontin, and subsequent chemiluminescent detection with the Renaissance kit from NEN (Boston, MA).

The antibody used for this procedure was a goat anti-osteopontin polyclonal antibody that is selective for rat osteopontin. This polyclonal antibody is termed OP-199 and was generated following essentially identical procedures to that described below in Example II for the anti-human osteopontin-selective goat polyclonal antibody, except that purified rat osteopontin was used as the immunogen instead of human osteopontin. The description of the generation of this antibody and its selectivity is further described in Liaw et al. Circ. Res. 74:214-224 (1994). The methods for making and preparing the rat osteopontin immunogen were also similar to those described below for human osteopontin.

A Renal biopsy was taken at day 17 and at the time of sacrifice (6 weeks). Osteopontin levels in rat tissues were examined by immunohistochemistry using the above goat anti-rat osteopontin-selective antibody. The extent of interstitial inflammation and fibrosis was determined histologically using methods well known to those skilled in the art and as described by Giachelli et al., Kidney Int. 45:515-524 (1994). The results of these histological analyses revealed that the extent and severity of the interstitial inflammation and fibrosis increased over time in the angiotensin II-induced hypertensive animals.

The results of the immunoblot assays described above are shown in Figure 2. Briefly, in untreated control animals, urinary levels of osteopontin were essentially undetectable. In contrast, in angiotensin II-induced hypertensive animals, there was a significant increase in osteopontin amounts so that detectable levels of this protein could be measured in each of the treated animals. These results, combined with the histological observations described above, indicate that urinary osteopontin levels concomitantly increase with the degree and progression of interstitial inflammation and fibrosis.

EXAMPLE II

Quantitative Immunoaffinity Assay for Osteopontin

This Example describes a sandwich ELISA for the sensitive and quantitative measurement of osteopontin.

To quantitate levels of osteopontin within a heterogeneous solution such as urine, a sandwich enzyme linked immunoaffinity assay was used that employed two

osteopontin-selective antibodies. The first antibody was used to selectively capture osteopontin from the urine and immobilize it onto a solid support. The second antibody, or detection antibody, was used as a quantitative measurement of the osteopontin immobilized onto the solid support. An enzyme linked secondary antibody or streptavidin was used to generate a colorimetric reaction which was quantitated by spectroscopy. The amount of bound osteopontin directly correlated with the amount of osteopontin in the sample.

Osteopontin-selective polyclonal and monoclonal antibodies have each been generated for use in the osteopontin ELISA detection system described above. Briefly, two polyclonal antibodies selective to human osteopontin have been generated and characterized. One of the polyclonal antibodies, termed OP-189, is a goat polyclonal antibody whereas the second polyclonal antibody, termed LF-7, was made in rabbit. The goat polyclonal is selective for both recombinant and purified forms of human osteopontin. Antibody LF-7 was provided by Dr. Larry Fisher at the National Institutes of Health (Bethesda, Maryland) and is also selective for both naturally purified and recombinant human osteopontin.

The methods used for generating each of the above polyclonal antibodies are well known in the art and are described in common laboratory manuals such as Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989). The osteopontin immunogen used for the production of the OP-189 polyclonal antibody was purified human urinary osteopontin. The selectivity of this antibody was determined by immunoblot, immunoprecipitation and ELISA

procedures against osteopontin in urine samples using purified osteopontin as a control.

Monoclonal antibodies have also been generated for use in the osteopontin detection system described above. Briefly, the monoclonals were generated by immunization of mice with purified recombinant human osteopontin. Hybridoma fusions were performed by the monoclonal Antibody Facility at the University of Washington according to well known and commonly accepted methods in the art. Again, such methods can be found described in common laboratory manuals such as Harlow et al., *supra*. Multiple screening of the fusions resulted in the identification of about 150 hybridomas that stained positive for recombinant human osteopontin antigen. Supernatants from two of these positive clones where studied further and were able to detect purified human urinary osteopontin in an immunoblot affinity assay. These two osteopontin-selective monoclonal antibodies were termed HYA10G8 and MSC9G9.

The purified recombinant or urinary osteopontin used in either of the above immunization or binding assay procedures was prepared using reagents and methods known to those skilled in the art. Purified human osteopontin was prepared from urinary sources by precipitation, essentially according to the method of Bohle et al., Am. J. Nephrol. 7:421-433 (1987) and Liaw et al., *supra*, 1994. Briefly, urine was dialyzed against buffer A (10 mM phosphate buffer, pH 7.0; 150 mM NaCl) at 4°C in the presence of protease inhibitors (0.3 µM aprotinin, 1 µM leupeptin, and 100 µM PMSF), and the sample centrifuged at 10,000 x g for 15 minutes. An anion exchange column was equilibrated with buffer A, and sample applied to the column at 0.5 ml per minute. The column was washed with

buffer A and then eluted with a gradient of NaCl (0.15 M-1.0 M) in phosphate buffer, pH 7.0. Column fractions were subsequently analyzed by ELISA, and fractions corresponding to the osteopontin peak pooled. A 1/10 volume of sodium citrate solution (3.8 g/100 ml H_2O) and 1/10 volume of barium chloride solution (15 g/100 ml H_2O) were added to the pooled fractions.

Osteopontin was precipitated for 48 to 72 hours at 4°C and centrifuged at 10,000 rpm for 15 minutes. The pellet was washed once with barium chloride solution, and twice with distilled water. Osteopontin was recovered by elution with a solution of 0.2 M sodium citrate, pH 6.8, and centrifugation for 2-3 minutes in a microcentrifuge at 4°C. The supernatant containing osteopontin was transferred to a clean tube.

Full-length recombinant human osteopontin was expressed as a histidine-tagged protein and purified as described in Smith et al., J. Biol. Chem. 271:28485-28491 (1996). Briefly, an expression plasmid containing histidine-tagged osteopontin (His-OPN) was generated by cloning a polymerase chain reaction fragment containing the full-length splice variant of human osteopontin (OP10; Young et al., *supra*, 1990) into the BamHI site of vector pQE30 (Qiagen, Chatsworth, CA). *E. coli* transformed with the His-OPN plasmid was grown in LB with 100 μg/ml ampicillin and induced with isopropyl-1-thio-β-D-galactopyranoside at 37°C to express the histidine-tagged protein. Tagged protein was subsequently purified from bacterial cells according to the manufacturer's instructions using the Q/Aexpressionist kit (Qiagen), chromatographed on Ni-nitrilotriacetic acid resin, and eluted with 0.2 M

imidazole. The recombinant histidine-tagged osteopontin was analyzed by SDS-PAGE.

The above described antibodies were used in a ELISA immunoaffinity binding assay formatted to first capture and immobilize osteopontin from the sample and then to quantitate the amount of bound osteopontin in a second detection step. Briefly, multi-well ELISA plates (Nunc Maxisorp 96-well plates) were coated with 50 µl of goat anti-osteopontin-selective antibody (OP-189) at a concentration of 20 µg/ml in phosphate-buffered saline (1 µg/well). The plates were incubated with antibody for two hours at room temperature or overnight at 4°C and then washed with two five minute incubations of 350 µl per well phosphate-buffered saline. Unbound sites were blocked by the addition of 200 µl/well of a solution containing 2.0% bovine serum albumin (BSA) and 1.5% goat serum in phosphate-buffered saline and incubated for an additional two hours at room temperature. Following blocking, the wells were again washed twice with two five minute incubations of 350 µl of phosphate-buffered saline. Plates coated with an osteopontin-selective capture antibody and blocked as described above can be used directly for sample measurements or, alternatively, stored at 4°C.

Sample determinations were performed by the addition of 50 µl/well of osteopontin-containing samples. A standard curve consisting of known amounts of osteopontin was constructed in parallel for quantitation of osteopontin in the sample. The amount of osteopontin used for the standard curve consisted of 1, 2, 4, 8, 16, 32, or 64 ng/well of purified human osteopontin. The osteopontin-containing sample and the purified standards were incubated for two hours at room temperature or

overnight at 4°C followed by three five minute washes with phosphate-buffered saline.

For the detection of osteopontin bound by the immobilized capture antibody, 100 µl of rabbit anti-human osteopontin antibody LF-7 was added at a dilution of 1:1000 in the blocking solution described above. Alternatively, the previously described monoclonal antibodies (HYA10G8 and MSC9G9) can be substituted at a similar dilution. The osteopontin-selective detection antibody was incubated for one hour at room temperature followed by three five minute washes with phosphate-buffered saline.

A biotinylated secondary antibody was used to quantitate the amount of osteopontin-bound detection antibody. Briefly, 100 µl of secondary biotinylated goat anti-rabbit antibody was used at a dilution of 1:200 in blocking solution (or alternatively, horse anti-mouse antibody is used if osteopontin-selective monoclonal antibodies are employed in the detection step described above; Vector Laboratories; Burlingame CA). The secondary antibody was incubated for one hour at room temperature followed by three five minute washes in phosphate-buffered saline. For colorimetric detection of the bound secondary antibody, 50 µl of ABC solution from Vectastain Elite ABC kit was prepared and used according to the manufacturer's instructions (Vector Laboratories). Following a 45 minute incubation at room temperature the wells were again washed three times with phosphate-buffered saline.

Development of the stain proceeded by the addition of 150 µl of chromogenic solution (10 mg of o-phenylenediamine in 10.0 ml of 0.1 M sodium citrate, pH

4.5 with 5 ml of 30% H_2O_2) and reactions were stopped by adding 100 µl of 4.5 M sulfuric acid to each well. Absorbance was read on a microplate reader at 490 nm (BIO-TEK Instrument EL 312e; Bio-Tek Instruments; Winooski VT).

The results of this assay are shown in Figure 3. Briefly, Figure 3A shows a standard curve using recombinant human osteopontin as antigen whereas Figure 3B shows a standard curve using purified human urinary osteopontin. These results indicate a detection sensitivity of at least 0.2-1.5 µg/ml of soluble osteopontin. Moreover, both recombinant (Figure 3A) and human urinary-derived osteopontin (Figure 3B) yielded similar detection curves and is therefore applicable for the quantitative detection of osteopontin concentration in urine samples. However, when used for the quantitation of urinary levels of osteopontin, measurements are normalized to urinary creatinine concentrations to correct for effects of concentration or dilution of urine.

EXAMPLE III

ELISA Assay of Human Osteopontin Concentration in Normal Urine and Urine of Patients with Renal Damage

This example describes the determination of osteopontin concentration in normal urine and in urine of patients with renal damage.

Osteopontin concentrations in human urine samples were determined by an ELISA similar to the method described in Example II. Various osteopontin antibodies were used in the assay. The rabbit polyclonal antibodies LF-7, LF-123 and LF-124, provided by Dr. Larry Fisher of

the National Institutes of Health, were used to initially capture and bind osteopontin in a sample to the wells of a plate. Another osteopontin antibody, the goat polyclonal antibody OP-189, was used to detect the osteopontin captured on the wells of the plate.

Briefly, 100 µl per well of rabbit anti-human osteopontin antibody LF-7, diluted 1:1000 in 0.1 M sodium bicarbonate, pH 9.0, was coated onto Nunc Maxisorp plates (Nalge Nunc; Rochester NY). The plates were incubated at 4°C overnight for 16 hr. Plates were washed 2 times for 5 min with 350 µl per well of 10 mM Tris, pH 8.0, 150 mM NaCl and 0.05% Tween-20 (ST-Tween). The wells were blocked with 200 µl per well of 1% BSA in ST-Tween for 2 hr at room temperature. The wells were then washed 3 times for 5 min per wash with ST-Tween.

Known concentrations of recombinant osteopontin, which has a His molecular tag (see Example II), or urine samples were diluted in 1% BSA in ST-Tween, and 50 µl of dilutions were added to individual wells. Recombinant osteopontin was diluted to give 4, 8, 16, 32 and 64 ng/well. Diluted standards or urine samples were incubated at 37°C for 1 hr. Plates were washed 3 times with ST-Tween, and 100 µl of goat anti-human osteopontin antibody (OP-189), diluted to 40 µg/ml in 1% BSA in ST-Tween, was added to the wells and incubated at 37°C for hr. After washing 3 times for 5 min with ST-Tween, 100 µl of biotinylated anti-goat antibody (Vector BA-5000; Vector Laboratories; Burlingame CA), at a 1:200 dilution in 1% BSA in ST-Tween, was added to each well and incubated at 37°C for 1 hr. Wells were washed 3 times for 5 min with ST-Tween. A 50 µl aliquot of ABC solution (Vectastain Elite ABD Kit; Vector PK-6100; Vector Laboratories) was added to each well and incubated for 45

min at room temperature, followed by three 5 min washes with ST-Tween. A chromagenic solution of o-phenylenediamine was prepared by adding one tablet of phenylenediamine (Sigma P-8287; Sigma; St. Louis MO) to 10 ml of 0.1 M sodium citrate, pH 4.5, containing 5 µl of 30% H_2O_2, and 150 µl of chromogenic solution was added to each well and incubated to allow color development as suggested by the manufacturer. The reaction was stopped by adding 100 µl of 4.5 M sulfuric acid to each well, and the absorbance at 490 nm was measured.

Urine samples were collected from normal individuals and from patients having renal damage. In initial studies, the osteopontin concentration in human urine was found to be stable for at least several days and stable to freezing, thawing and refreezing.

A standard curve was generated using known concentrations of osteopontin. Briefly, a recombinant form of osteopontin was used as a standard (see Example II). A series of two-fold dilutions of a known concentration of recombinant osteopontin was assayed. The concentration ranged from 0.08 µg/ml to 1.28 µg/ml. An example of a standard curve using recombinant osteopontin is shown in Figure 4. The linear range of the assay was between 0.16 µg/ml and 0.64 µg/ml. The standard curve was calculated using the formula $Y = (A-D/1+(X/C)^B)+D$; where A = 0.030; B = 2.331; C = 33.472; and D = 0.562. No weighting function was used. Values and ranges were determined to be: Min(X) = 4,000; Max(X) = 64,000; Min(Y) = 0.034; Max(Y) = 0.465; SSE = 7.436E-06; and RMS = 0.003, where SSE is "sum of squares of the errors" and RMS is "root mean square."

To assay urine samples, two-fold dilutions of urine, starting with a 1:10 dilution, were made in 1% BSA/ST-Tween. Dilutions were assayed and compared to the standard curve of known osteopontin levels. Quantitation of osteopontin levels in urine were based on dilutions that fell in the linear range of the standard curve.

Using such an assay with recombinant osteopontin as the standard, the osteopontin level in normal urine was determined to be about 4 to 6 µg/ml. In contrast, osteopontin levels in urine samples from patients with renal damage were about 5 times greater, ranging between 20 to 30 µg/ml. These results were obtained with a standard error of less than 10%. Figure 4 shows the positions on the osteopontin standard curve that normal urine and urine from patients with renal damage occurs, indicating the range of osteopontin concentration in human urine samples.

These results demonstrate that the ELISA assay can be used to measure the concentration of osteopontin in human urine samples. These results also indicate that a urine concentration of osteopontin above levels found in normal individuals is diagnostic for renal pathologies in a patient.

Throughout this application various publications have been referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We Claim:

1. A method of diagnosing a renal pathology, comprising determining a concentration of urinary osteopontin in a sample isolated from an individual having or suspected of having a renal pathology and comparing said determined concentration to a urinary osteopontin concentration from a normal individual, wherein said determined concentration of urinary osteopontin in said sample indicates the presence or absence of said renal pathology.

2. The method of claim 1, wherein an increase in said determined concentration of urinary osteopontin in said sample compared to said urinary osteopontin concentration from a normal individual indicates the presence of said renal pathology.

3. The method of claim 1, wherein an unchanged concentration of urinary osteopontin in said sample compared to said urinary osteopontin concentration from a normal individual indicates the absence of a renal pathology.

4. The method of claim 1, wherein said renal pathology is selected from a group of renal pathologies consisting of tubulointerstitial inflammation, tubulointerstitial injury, glomerulonephritis, polycystic kidney disease, hypertensive renal damage, diabetes, renal ischemia, ureteral obstruction, cyclosporine toxicity, age-related renal pathologies, renal fibrosis and renal failure.

5. The method of claim 1, wherein said individual is a human.

6. The method of claim 1, wherein said urinary osteopontin concentration is determined by an affinity binding assay.

7. The method of claim 6, wherein said affinity binding assay is an immunoaffinity binding assay.

8. The method of claim 2, wherein said urinary osteopontin concentration in said sample isolated from said individual having or suspected of having a renal pathology is greater than about 5 µg/ml, preferably greater than about 10-30 µg/ml, more preferably greater than about 40 µg/ml.

9. The method of claim 2, wherein said urinary osteopontin concentration in said sample isolated from said individual having or suspected or having a renal pathology is greater than about 20 µg/ml.

10. A method of predicting the onset of a renal pathology, comprising determining an increased concentration of urinary osteopontin in a sample isolated from an individual having or suspected or having a renal pathology compared to a sample isolated from a normal individual, wherein said increased concentration of urinary osteopontin in said sample indicates the onset of said renal pathology.

11. The method of claim 10, wherein said renal pathology is selected from a group of renal pathologies consisting of tubulointerstitial inflammation, tubulointerstitial injury, glomerulonephritis, polycystic kidney disease, hypertensive renal damage, diabetes, renal ischemia, ureteral obstruction, cyclosporine

toxicity, age-related renal pathologies, renal fibrosis and renal failure.

12. The method of claim 10, wherein said individual is a human.

13. The method of claim 10, wherein said urinary osteopontin concentration is determined by an affinity binding assay.

14. The method of claim 13, wherein said affinity binding assay is an immunoaffinity binding assay.

15. The method of claim 10, wherein said urinary osteopontin concentration in said sample isolated from said individual having or suspected of having a renal pathology is greater than about 5 µg/ml, preferably greater than about 10-30 µg/ml, more preferably greater than about 40 µg/ml.

16. The method of claim 10, wherein said urinary osteopontin concentration in said sample isolated from said individual having or suspected or having a renal pathology is greater than about 20 µg/ml.

17. A method of monitoring the progression of a renal pathology, comprising the steps of:

(a) determining the concentration of urinary osteopontin in a first sample isolated from an individual having or suspected of having a renal pathology;

(b) determining the concentration of urinary osteopontin in a temporally independent second sample isolated from said individual; and

(c) comparing the concentration of urinary osteopontin in said first and second samples from said individual, wherein an increase in the osteopontin concentration between said first and said second samples is indicative of an increased progression of the renal pathology and wherein a decrease in the osteopontin concentration between said first and second samples is indicative of a decreased progression of said renal pathology.

18. The method of claim 17, wherein said renal pathology is selected from a group of renal pathologies consisting of tubulointerstitial inflammation, tubulointerstitial injury, glomerulonephritis, polycystic kidney disease, hypertensive renal damage, diabetes, renal ischemia, ureteral obstruction, cyclosporine toxicity, age-related renal pathologies, renal fibrosis and renal failure.

19. The method of claim 17, wherein said individual is a human.

20. The method of claim 17, wherein said urinary osteopontin concentration is determined by an affinity binding assay.

21. The method of claim 20, wherein said affinity binding assay is an immunoaffinity binding assay.

22. The method of claim 17, wherein said increase in said urinary osteopontin concentration in said samples isolated from said individual having or suspected of having a renal pathology is greater than about 5 µg/ml, preferably greater than about 10 µg/ml, more preferably greater than about 30 µg/ml between said first and second samples.

23. The method of claim 17, wherein said increase in said urinary osteopontin concentration in said sample from said individual having or suspected or having a renal pathology is greater than about 20 µg/ml between said first and second samples.

24. The method of claim 17, wherein said decrease in said urinary osteopontin concentration in said samples isolated from said individual having or suspected of having a renal pathology is less than about 5 µg/ml, preferably less than about 10 µg/ml, more preferably less than about 30 µg/ml between said first and second samples.

25. The method of claim 17, wherein said decrease in said urinary osteopontin concentration in said sample from said individual having or suspected of having a renal pathology is less than about 20 µg/ml between said first and second samples.

26. A method of diagnosing a renal pathology, comprising the steps of:

a) contacting an immobilized first osteopontin-selective antibody with a urine sample isolated from an individual having or suspected of having a renal pathology under conditions which allow binding of

osteopontin to said immobilized osteopontin-selective antibody so as to immobilize osteopontin in said urine sample;

b) contacting said immobilized osteopontin with a second osteopontin-selective antibody under conditions which allow binding of said second osteopontin-selective antibody to said immobilized osteopontin; and

c) determining the amount of bound osteopontin to said second osteopontin-selective antibody, wherein an increased amount of bound osteopontin from said urine of said individual having or suspected of having a renal pathology compared to bound osteopontin from urine of a normal individual indicates the presence of a renal pathology.

27. The method of claim 26, wherein said renal pathology is selected from a group of renal pathologies consisting of tubulointerstitial inflammation, tubulointerstitial injury, glomerulonephritis, polycystic kidney disease, hypertensive renal damage, diabetes, renal ischemia, ureteral obstruction, cyclosporine toxicity, age-related renal pathologies, renal fibrosis and renal failure.

28. The method of claim 26, wherein said individual is a human.

29. The method of claim 26, wherein said bound urinary osteopontin from said sample isolated from said individual having or suspected or having a renal pathology corresponds to a urine concentration greater

than about 5 µg/ml, preferably greater than about 10-30 µg/ml, more preferably greater than about 40 µg/ml.

30. The method of claim 26, wherein said urinary osteopontin from said sample isolated from said individual having or suspected or having a renal pathology corresponds to a urine concentration greater than about 20 µg/ml.

31. A kit for diagnosing a renal pathology, comprising an osteopontin-selective antibody, osteopontin and an ancillary reagent.

32. The kit of claim 31, wherein said osteopontin-selective antibody is the monoclonal antibody HYA10G8 or MSC9G9.

33. The kit of claim 31, further comprising a second osteopontin-selective antibody.

34. The kit of claim 31, wherein said ancillary reagent is selected from the group of ancillary reagents consisting of a detectable osteopontin-selective antibody, a detectable secondary binding molecule, a blocking solution, wash buffer, binding buffer, detectable substrate, detectable substrate buffer, dilution buffer.

35 The kit of claim 31, further comprising written instructions for detecting urinary osteopontin concentrations.

36. An osteopontin-selective antibody comprising a detectable moiety.

37. An osteopontin-selective monoclonal antibody selected from the group consisting HYA10G8 or MSC9G9.

```
                .                   .                   .                   .
1   GAG GCA GCA GCA GGA GGA GGC AGA GAC AGC ATC GTC GGG ACC AGA CTC GTC TCA GGC
                .                   .                   .                   .
61  CAG TTG CAG CCT TCT CAG CCA AAC GCC GAC CAA GGA AAA CTC ACT ACC ATG AGA ATT GCA
                                                          Met Arg Ile Ala
                .                   .                   .                   .
121 GTG ATT TGC TTT TGC CTC CTA GGC ATC ACC TGT GCC ATA CCA GTT AAA CAG GCT GAT TCT
4   Val Ile Cys Phe Cys Leu Leu Gly Ile Thr Cys Ala Ile Pro Val Lys Gln Ala Asp Ser
                .                   .                   .                   .
181 GGA AGT TCT GAG GAA AAG CAG CTT TAC AAC AAA TAC CCA GAT GCT GTG GCC ACA TGG CTA
25  Gly Ser Ser Glu Glu Lys Gln Leu Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu
                .                   .                   .                   .
241 AAC CCT GAC CCA TCT CAG AAG CAG AAT CTC CTA GCC CCA CAG ACC CTT CCA AGT AAG TCC
45  Asn Pro Asp Pro Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Thr Leu Pro Ser Lys Ser
                .                   .                   .                   .
301 AAC GAA AGC CAT GAC CAC ATG GAT GAT ATG GAT GAA GAT GAT GAC CAT GTG GAC
65  Asn Glu Ser His Asp His Met Asp Asp Met Asp Glu Asp Asp Asp His Val Asp
                .                   .                   .                   .
361 AGC CAG GAC TCC ATT GAC TCG AAC GAC TCT GAT GAT GTA GAT GAC ACT GAT GAT TCT CAC
85  Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp Val Asp Asp Thr Asp Asp Ser His
                .                   .                   .                   .
421 CAG TCT GAT GAG TCT CAC CAT TCT GAT GAA TCT GAT GAA CTG GTC ACT GAT TTT CCC ACG
105 Gln Ser Asp Glu Ser His His Ser Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr
                .                   .                   .                   .
480 GAC CTG CCA GCA ACC GAA GTT TTC ACT CCA GTT GTC CCC ACA GTA GAC ACA TAT GAT GGC
125 Asp Leu Pro Ala Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly
```

FIG. IA

SUBSTITUTE SHEET (RULE 26)

```
540   CGA GGT GAT AGT GTG GTT TAT GGA CTG AGG TCA AAA TCT AAG AAG TTT CGC AGA CCT GAC
145   Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe Arg Arg Pro Asp

601   ATC CAG TAC CCT GAT GCT ACA GAC GAG GAC ATC ACC TCA CAC ATG GAA AGC GAG GAG TTG
165   Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr Ser His Met Glu Ser Glu Glu Leu

661   AAT GGT GCA TAC AAG GCC ATC CCC GTT GCC CAG GAC CTG AAC GCG CCT TCT GAT TGG GAC
185   Asn Gly Ala Tyr Lys Ala Ile Pro Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp

721   AGC CGT GGG AAG GAC AGT TAT GAA ACG AGT CAG CTG GAT GAC CAG AGT GCT GAA ACC CAC
205   Ser Arg Gly Lys Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His

781   AGC CAC AAG CAG TCC AGA TTA TAT AAG CGG AAA GCC AAT GAT GAG AGC AAT GAG CAT TCC
225   Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser Asn Glu His Ser

841   GAT GTG ATT GAT AGT CAG GAA CTT TCC AAA GTC AGC CGT GAA TTC CAC AGC CAT GAA TTT
245   Asp Val Ile Asp Ser Gln glu Leu Ser Lys Val Ser Arg Glu Phe His Ser His Glu Phe

901   CAC AGC CAT GAA GAT ATG CTG GTT GTA GAC CCC AAA AGT AAG GAA GAA GAT AAA CAC CTG
265   His Ser His Glu Asp Met Leu Val Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu

961   AAA TTT CGT ATT TCT CAT GAA TTA GAT AGT GCA TCT TCT GAG GTC AAT TAA AAG GAG AAA
285   Lys Phe Arg Ile Ser His Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
```

FIG. 1B

```
1021  AAA TAC AAT TTC TCA CTT TGC ATT TAG TCA AAA GAA AAA ATG CTT TAT AGC AAA ATG AAA

1081  GAG AAC ATG AAA TGC TTC TTT CTC AGT TTA TTG GTT GAA TGT GTA TCT ATT TGA GTC TGG

1141  AAA TAA CTA ATG TGT TTG ATA ATT AGT TTA GTT TGT GGC TTC ATG GAA ACT CCC TGT AAA

1201  CAA AAG CTT CAG GGT TAT GTC TAT GTT CAT TCT ATA GAA GAA ATG CAA ACT ATC ACT GTA

1261  TTT TAA TAT TTG TTA TTC TCT CAT GAA TAG AAA TTT ATG TAG AAG CAA ACA AAA TAC TTT

1321  TAC CCA CTT AAA AAG AGA ATA TAA CAT TTT ATG TCA CTA TAA TCT TTT GTT TTT TAA GTT

1381  AGT GTA TAT TTT GTT GTG ATT ATC TTT TGT GGT GTG AAT AAA TCT TTT ATC TTG AAT GTA

1441  ATA AGA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA
```

FIG. IC

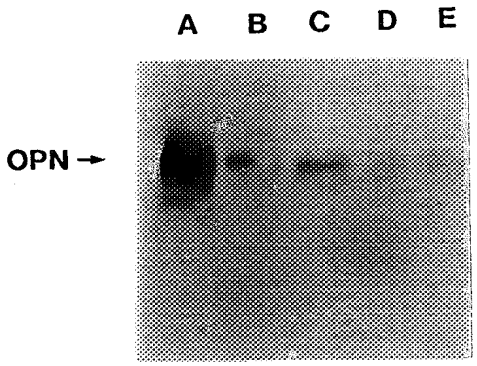


FIG.2

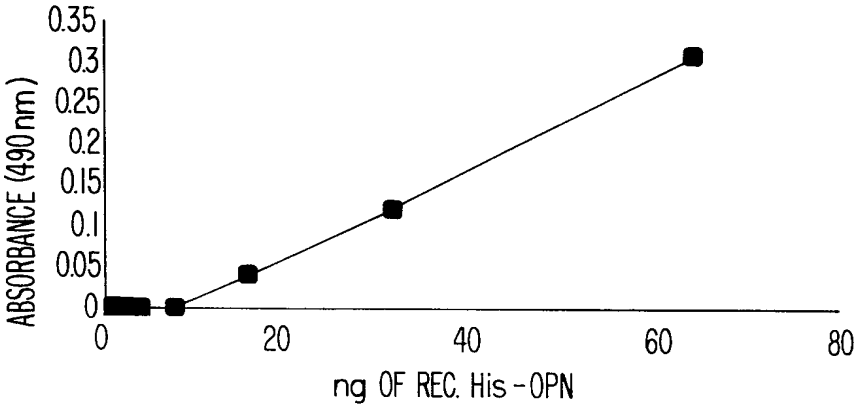

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ABSORBANCE (490 nm)
0
20
40
60
80
ng OF REC. His-OPN

FIG. 3A

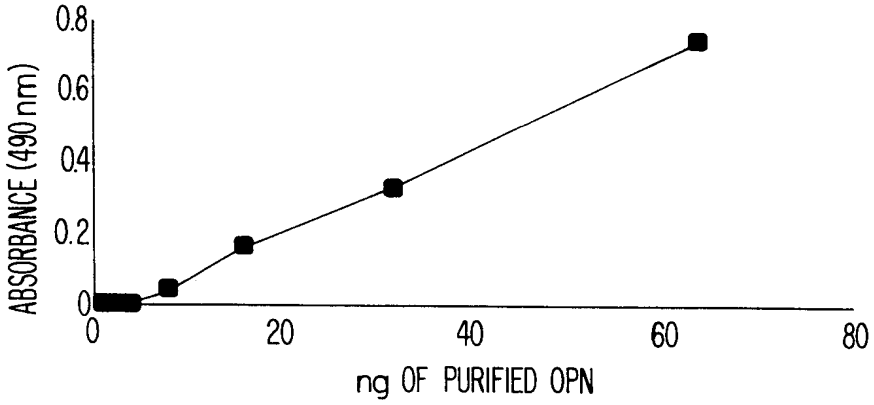

0.8
0.6
0.4
0.2
0
ABSORBANCE (490 nm)
0
20
40
60
80
ng OF PURIFIED OPN

FIG. 3B

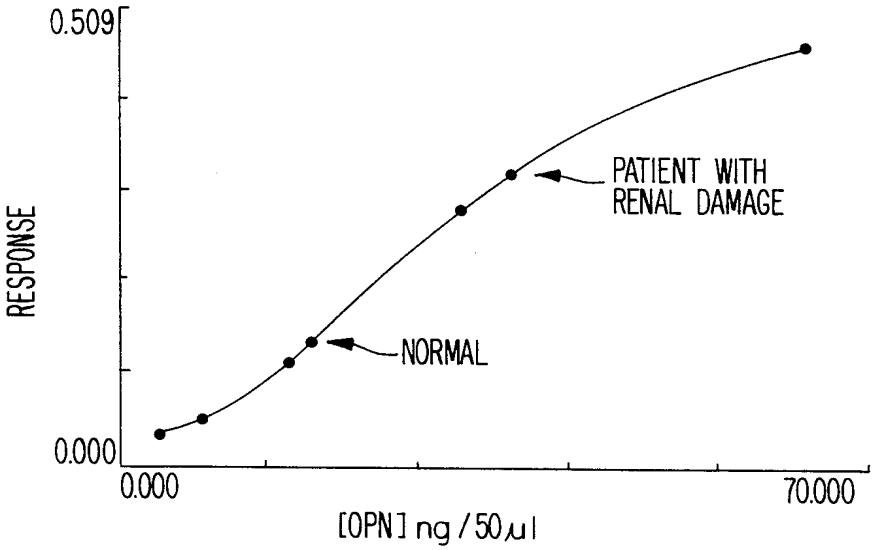

0.509
0.000
RESPONSE
PATIENT WITH
RENAL DAMAGE
NORMAL
0.000
70.000
[OPN] ng/50µl

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/20167

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C07K16/24 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 304 496 A (J. HOYER ET AL.) 19 April 1994 see column 4, line 42 - line 57; claims 3-8 see column 8, line 59 - column 9, line 54 --- -/--	1-37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 1999	15/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 125, no. 7, 12 August 1996 Columbus, Ohio, US; abstract no. 81107, XP002095283 cited in the application see abstract & D.S. BAUTISTA ET AL.: "Low-molecular-weight variants of osteopontin generated by serine proteinases in urine of patients with kidney stones. " JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 61, no. 3, 1996, pages 402-409, London UK	1-37
A	MEDLINE, Washington DC USA; abstract no. 97440611, see abstract XP002095282 & A.B. MAGIL ET AL.: "Osteopontin in chronic puromycin aminonucleoside nephrosis." JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 8, no. 9, 1 September 1987, pages 1383-1390, Washington DC USA	1-37
X	CHEMICAL ABSTRACTS, vol. 123, no. 25, 18 December 1995 Columbus, Ohio, US; abstract no. 336963, XP002095284	36
A	see abstract & D.S. BAUTISTA ET AL.: "A monoclonal antibody against inhibins RGD-mediated cell adhesion to osteopontin." ANNALES OF THE NEW YORK ACADEMY OF SCIENCES, vol. 760, 1995, pages 309-311, New York NY USA	37

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/20167

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5304496 A	19-04-1994	AU 2186392 A	12-01-1993
		WO 9222316 A	23-12-1992